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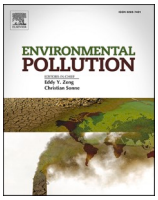

Effect of an antidepressant on aquatic ecosystems in the presence of microplastics: A mesocosm study☆

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A B S T R A C T

Emerging pollutants, such as pharmaceuticals and microplastics have become a pressing concern due to their widespread presence and potential impacts on ecological systems. To assess the ecosystem-level effects of these pollutants within a multi-stressor context, we simulated real-world conditions by exposing a near-natural multi-trophic aquatic food web to a gradient of environmentally relevant concentrations of fluoxetine and microplastics in large mesocosms over a period of more than three months. We measured the biomass and abundance of different trophic groups, as well as ecological functions such as nutrient availability and decomposition rate. To explore the mechanisms underlying potential community and ecosystem-level effects, we also performed behavioral assays focusing on locomotion parameters as a response variable in three species: *Daphnia magna* (zooplankton prey), *Chaoborus flavicans* larvae (invertebrate pelagic predator of zooplankton) and *Asellus aquaticus* (benthic macroinvertebrate), using water from the mesocosms. Our mesocosm results demonstrate that presence of microplastics governs the response in phytoplankton biomass, with a weak non-monotonic dose-response relationship due to the interaction between microplastics and fluoxetine. However, exposure to fluoxetine evoked a strong non-monotonic dose-response in zooplankton abundance and microbial decomposition rate of plant material. In the behavioral assays, the locomotion of zooplankton prey *D. magna* showed a similar non-monotonic response primarily induced by fluoxetine. Its predator *C. flavicans,* however, showed a significant non-monotonic response governed by both microplastics and fluoxetine. The behavior of the decomposer *A. aquaticus* significantly decreased at higher fluoxetine concentrations, potentially leading to reduced decomposition rates near the sediment. Our study demonstrates that effects observed upon short-term exposure result in more pronounced ecosystem-level effects following chronic exposure.

☆ This paper has been recommended for acceptance by Wen Chen.

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1. Introduction

Global anthropogenic degradation of water bodies has been characterized by increased occurrence of stressors, such as contaminants of emerging concern, persisting in aquatic systems (Schwarzenbach et al., 2006; Burkhardt-Holm, 2010). Particularly, pharmaceuticals and microplastics have received great attention in recent years due to their increasing concentrations in aquatic ecosystems. Regulatory and policy interest focuses on defining standard environmental risk assessment metrics such as effective concentration, lethal concentrations and no-observed effect concentrations on a maximum of 3-trophic levels (European Medicines Agency, 2018). However, this does not provide a complete understanding about the risk posed by these stressors on aquatic organisms and interactions that are key to ecosystem functioning (Bertram et al., 2022).

Approaches like effect-based assays and predictive models are currently being considered to understand aquatic toxicity of chemical mixtures on a single species (Altenburger et al., 2000; Schuijt et al., 2021). However, it is essential to be able to predict change driven by multiple stressors, beyond single species dynamics in isolation, in order to understand the complex interactions and potential risks in aquatic ecosystems (Hamid et al., 2021; Laskowski et al., 2010). In addition, conventional ecotoxicology tests focus on a straightforward, linear, or monotonic nonlinear relationship between dose and response. However, some studies have shown that the effects of certain pharmaceuticals such as antidepressants can be intricate and non-monotonic, resulting in varied outcomes depending on the dose or duration of exposure (Vandenberg et al., 2012). Several theories have been proposed to explain these relationships, such as receptor saturation, hormesis, or biphasic physiological responses (Agathokleous & Calabrese, 2019; Calabrese, 2013). To accurately evaluate the risks linked to emerging contaminants it is imperative to assess capacity for non-monotonic responses across a dose gradient.

Emerging contaminants such as pharmaceuticals remain chemically active at low concentrations resulting in many physiological and behavioral effects on aquatic biota (see reviews Brodin et al., 2014; Van Donk et al., 2016). Antidepressants are among the most commonly found pharmaceuticals in aquatic ecosystems (Ford & Fong, 2016). Fluoxetine, an antidepressant belonging to selective serotonin reuptake inhibitors (SSRIs), is used to treat depression and compulsive behaviors in humans by regulating serotonin reuptake and has been detected in surface waters at concentrations ranging from 2.5 ng/L to 109.2 ng/L (Gardner et al., 2012; Schultz & Furlong, 2008). Both vertebrates and invertebrates use serotonin as a neurotransmitter (Tierney, 2020), leading to species-specific effects on behavior and physiology, depending on the dose concentration. Fluoxetine can alter behavioral endpoints in aquatic organisms such as locomotion, phototaxis, geotaxis, aggression, and predation in varying manners (Guler & Ford, 2010; Labaude et al., 2015; McCallum et al., 2017; Thoré et al., 2020, 2023). SSRIs can demonstrate complex, non-monotonic relationships between dose and response (Bossus et al., 2014; Ford and Fong, 2016; Guler and Ford, 2010). Furthermore, the capacity of antidepressants to induce these non-monotonic dose-response relationships varies broadly across the literature (Sumpter et al., 2014). Lack of understanding of the full spectrum of dose specific responses could result in an underestimation of harmful effects at lower concentrations and thus poor ecological impact assessments (Hill et al., 2018).

Microplastics are another class of emerging contaminants that have been widely recognized for their potential adverse effects on aquatic biota such as through food dilution, internal and external physical damage, oxidative stress and increased mortality (de Ruijter et al., 2020; Thornton-Hampton, et., 2022). There is limited data regarding the effect of pharmaceuticals (including fluoxetine) in absence versus presence of microplastics on trophic interactions or ecosystem functioning at environmentally relevant concentrations and exposure times. Nonetheless, ingestion of microplastics, as well as exposure to fluoxetine, have been shown to cause changes in zooplankton abundance (both increases and decrease), disruptions in trophic interactions, and alterations in behavior (Feijão et al., 2020; Nielsen & Roslev, 2018). It cannot be ruled out that interactions between fluoxetine, microplastics, and aquatic organisms can occur during long term exposure in freshwater ecosystems, either because of fluoxetine driven effects (Feijão et al., 2020), or microplastics driven effects (Kong & Koelmans, 2019), or both (Almeida et al., 2019).

Aquatic systems are a cocktail of stressors and generalized descriptions of effects focusing on a single stressor and on a single species do not convey the complexity of natural systems. There is thus a need for investigations with multiple species under a realistic multiple stressor scenario (Sumpter et al., 2014). Environmentally relevant testing systems such as mesocosms can be a valuable tool to address complex environmental scenarios in a controlled manner (Rosi-Marshall & Royer, 2012; Velthuis et al., 2017; Redondo-Hasselerharm et al., 2020). Mesocosm experiments can be maintained across a longer time period which better represent natural ecosystem dynamics in comparison to the short exposure durations of most experimental ecotoxicology (Bertram et al., 2022). This allows for the cycling and interaction of microplastics and fluoxetine across different compartments of the ecosystem (e.g. sediment vs water), affecting multiple trophic levels. The continued presence of these pollutants, whether in sediment, the water column, or within organisms, signifies persistent exposure and enables us to assess direct and indirect impacts at the food web and ecosystem level.

Using long-term indoor mesocosms systems, we aimed to assess the impact of fluoxetine on aquatic food-web structuring and functioning. In order to maximize relevance with respect to the current widespread presence of microplastics background levels (Koelmans et al., 2019), the experiments were performed in the presence and absence of such a background level of microplastics. We followed the nature of the response of phytoplankton biomass, zooplankton abundance and community composition, nutrient availability and decomposition of plant material to a gradient of fluoxetine concentration with background levels of microplastics. To further enhance our understanding about the mechanisms that might underlie potential community and ecosystem level effects, we additionally tested the treatment's effect on the locomotion behavior of naive individuals from three species i.e., *Chaoborus flavicans* larvae (invertebrate pelagic predator of zooplankton), *Daphnia magna* (zooplankton prey) and *Asellus aquaticus* (a benthic macroinvertebrate) representing ubiquitous genera in Dutch ponds and lake systems, covering different trophic levels and aquatic compartments (i.e. benthic and pelagic) by exposing them to water from the experimental mesocosms. As these selected species span multiple trophic levels—primary consumers, secondary consumers, and detritivores—and inhabit distinct aquatic compartments, understanding their behavioral changes can provide us with insights into some of the mechanisms underlying the observed ecosystem dynamics, such as trophic interaction strength and metabolic processes.

2. Methodology

2.1. Fluoxetine and microplastics

Fluoxetine hydrochloride (CAS No.56296-78-7) from SIGMA ALDRICH (The Netherlands) was used to prepare the stock solutions needed for the experimental treatments. Fluorescent spherical polystyrene (PS) microplastic spheres (10.23 μm ± 0.13 s d) from microparticles (Germany) were used. We choose PS microplastics as this polymer represents the average density of environmental microplastics (Redondo-Hasselerharm et al., 2018). Furthermore, PS particles are one of the most abundant polymers in the environment, just after polyethylene and polypropylene (Koelmans et al., 2019). Because using environmentally realistic microplastics would have added another level of complexity in understanding the effects observed, we opted for spherical particles as a proxy for microplastics. Microplastics were

extensively characterized and effects of plastic-associated chemicals were excluded. For further details regarding Quality Assurance and Quality Control (QA/QC) (de Ruijter et al., 2020) the reader is referred to the Supplementary materials. Both fluoxetine and microplastics stock solutions were prepared in demineralized water and microplastics stocks were stirred for 1 h prior to use to assure an adequate level of homogeneity of the dispersion.

2.2. Indoor mesocosms

2.2.1. Experimental design

We conducted experiments in nine indoor mesocosms, known as Limnotrons, each with a 922 L capacity (0.97 m diameter, depth ranging from 1.32 m to 1.37 m) (Velthuis et al., 2017; Verschoor et al., 2003). Our approach employed a regression design, with eight Limnotrons exposed to varying environmentally realistic concentrations, while one served as a control. These treatments included a single dose microplastics-only treatment and seven MP + fluoxetine treatments (see Fig. 1). Exposure spanned from March to June 2019, lasting 103 days.

Initially, all Limnotrons were filled with 845 L of pond water and 40 L of sediment from a shallow pond at the Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, on February 28, 2019 (T0). On the same day, the ambient sediment (except for the control) was spiked with environmentally realistic concentrations of fluoxetine (19 ng/g dry sediment) and microplastics (0.31 ng/g dry sediment) (Redondo-Hasselerharm et al., 2023). Pond water was then added to all mesocosms, and after a 24-h settling period, we introduced microplastics and fluoxetine into the water column, marking the first sampling (T_1) day. Microplastics (11.1 ng/L) were added once to all treatments (except the control) to achieve environmentally relevant concentrations in freshwater ecosystems (Koelmans et al., 2019). In contrast, fluoxetine concentrations varied (10, 15, 25, 50, 100, 250, and 500 ng/L; see Fig. 1), and fluoxetine was recharged into the Limnotrons 5 consecutive days per week throughout the 103-day experiment. This concentration range included environmental realistic exposures (Gardner et al., 2012; Schultz & Furlong, 2008) as well as slightly higher concentrations to capture potential non-monotonic dose-response relationships (Raman et al., 2024). Our design accounts for the different properties of microplastics (persistent) and fluoxetine (labile), representing environmentally realistic conditions and enabling the assessment of cumulative effects. To maintain fluoxetine presence at environmentally realistic conditions throughout the experiment, the Limnotrons received a 10 L replacement of aerated water dosed with fluoxetine corresponding to their treatment. Importantly, our aim was not to maintain a constant concentration of the starting conditions, but rather to achieve environmentally realistic concentrations. Both the choice of the fluoxetine gradients and the plastics are a balance between environmental realism and control. Given the nature of a long-term exposure experiment designed to mimic natural conditions, the fate of microplastics and fluoxetine within the experimental ecosystem involves multiple interactions and varying impacts across different compartments of the aquatic ecosystem.

Fluoxetine was assessed using solid phase extraction and QuEChERS procedures (Santos et al., 2016). The signal detection was below the background noise levels, and thus lower than the limit of quantification (LOQ). Fluoxetine degrades in the environment and has a relatively short half-lifetime of 1–4 days (Kwon & Armbrust, 2006), we therefore quantitatively modeled the actual fluoxetine concentration under our experimental conditions, see the Supplementary Materials. The sediment has a low background concentration of microplastics that is operationally defined as part of the designed constant background concentration. We emphasize that the sole purpose of adding microplastics was to ensure a more or less natural background concentration, not to interpret any observed effects in a dose-response context, where the dose must also be precisely known.

Temperature was regulated in all Limnotrons (20 °C ± 0.5 °C) using a custom-made climate control system (SpecView 32/859, SpecView Ltd., Uckfield, UK). Light intensity (175 ± 25 μmol photons/m^2/s) followed a 16:8 (light: dark) cycle, generated by HPS/MH lamps (CDM-TP Elite MW 315–400 W; AGRILIGHT B.V., Monster, The Netherlands). We monitored pH, electrical conductivity (μS/cm), and dissolved oxygen (mg/L) weekly using WTW Multi 350i Field meters. Additionally, we assessed the impact of microplastics and/or fluoxetine on microbial plant material decomposition. Weekly pooled water samples (10 L) were collected through three sampling ports at 7 cm (top), 62 cm (middle), and 117 cm (bottom) below the water surface. These samples were pooled for phytoplankton analysis, zooplankton determination, and nutrient analysis.

2.2.2. Phytoplankton accumulation and zooplankton abundance

Chlorophyll-a concentrations were determined in the 15 mL unfiltered subsample of the pooled samples by means of chlorophyll-a fluorescence using a Multiwavelength-excitation PAM fluorometry PHYTO-PAM (Heinz Walz Effeltrich, Germany). The parameters measured were: a) total chlorophyll-a concentration (μg chl-a/L), as proxy for biomass and b) effective quantum yield indicating phytoplankton cell fitness.

For zooplankton determination, 1 L subsamples from the 10 L pooled water samples were filtered over a 33 μm mesh, and the residue was

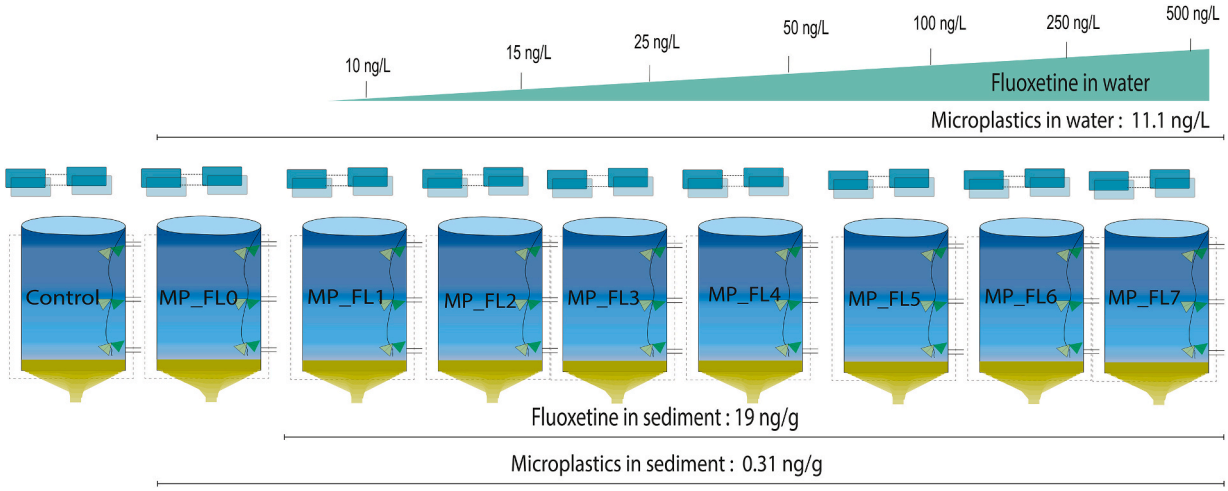

Fig. 1. Experimental setup and conditions of the indoor mesocosms used for this experiment. Concentration of fluoxetine (FL) and microplastics (MP) in both water and sediment in all the treatments are indicated at the top and bottom of the mesocosm diagrams, respectively.

fixed in Lugol's iodine solution and stored in the dark. Micro- and meso-zooplankton community abundance and composition were determined using a stereomicroscope (Leica WILD MZ8; Leica Microsystems, Wetzlar, Germany). All individuals present in samples were counted. Rotifers and Cladocerans were classified at genus level while adult Copepods were identified to order level. Copepod nauplii were included in the count without further taxonomic distinction.

2.2.3. Dissolved nutrient analysis and seston elemental composition

Subsamples of 50 mL from pooled samples were used to determine dissolved inorganic nutrients and seston elemental composition. The samples were filtered over pre-washed GF/F filters (Whatman), and 15 mL of filtrate was stored in −20 °C while the GF/F filters were dried in the oven at 60 °C for 24 h and stored in dark until determination. Following Velthuis et al., 2017, the concentration of dissolved nutrients (NO_2^-, NO_3^-, NH_4^+ and PO_4^{3-}) were determined in the 15 mL thawed samples by a QuAAtro Auto-Analyzer (Beun de Ronde B.V., Abcoude, NL). Particulate organic carbon and nitrogen were analyzed by FLASH 2000 NC elemental analyzer, (Interscience B.V., Breda, The Netherlands) and phosphorus was determined in the dried GF/F filters using QuAAtro Auto-Analyzer (Beun de Ronde B.V., Abcoude, NL).

2.2.4. Decomposition rate determination

Decomposition rate of plant litter over 90 days in our Limnotron treatments was determined using the Tea Bag Index (TBI) adapted for aquatic ecosystems (Seelen et al., 2019). A total of 3 Lipton green tea and 3 rooibos tea bags were deployed pairwise at three depths (i.e. top, middle and bottom) of each Limnotron to capture the activity of the decomposing microbial community in the entire mesocosm. Decomposition rates were determined by measuring the mass loss of the tea bags between initial mass and final mass following (Seelen et al., 2019).

2.3. Behavioral assays

Behavioral parameters measured in each assay were: distance moved (cm), velocity (cm/s), and duration of movement (s). To the best of our knowledge, these parameters are used for the first time in microplastics research, and represent crucial ecological processes (e.g., resource allocation and prey-predator interactions). As such, using behavioral endpoints allow for a better understanding of the sub-lethal impacts of pollutants, especially in a long-term multi-species system (Bertram et al., 2022). For example, velocity of movement and duration of movement are relevant in determining trophic interaction strength and distance moved and velocity can be used to make inferences regarding metabolism (South et al., 2019; Bertram et al., 2022).

Behavioral assays were carried out separately for *Chaoborus flavicans* (n = 6 per treatment), *Daphnia magna* (n = 12 per treatment) and *Asellus aquaticus* (n = 6 per treatment) representing ubiquitous genera across different trophic levels and distinct aquatic compartments (i.e. benthic, and pelagic) on day 5, day 7 and day 9, respectively. Depth integrated water samples were drawn from each Limnotron on the day of the assay, filtered through a mesh (33 μm) to remove any particles or organisms, and mixed immediately prior to the behavioral assays. Any mortality within the experimental period was recorded. *C. flavicans* and *A. aquaticus* assays were performed in flat bottomed 6 well microplates (35 mm diameter per well; volume 15 mL) and *D. magna* assays in 12 well microplates (22 mm diameter per well; volume 5 mL). One naive (non-prior exposed) individual was added to each well with 15 mL (for *C. flavicans* and *A. aquaticus*) and 5 mL (for *D. magna*) water from Limnotron treatments. The behavior of animals was assessed using a DanioVision® behavioral video tracking system with GigE digital camera at high resolution (1280 x 960 px) (Noldus® Information Technology, Wageningen, Netherlands) at a sampling rate of 25 frames per second. The video files were analyzed using EthoVision® XT software (Version 14; Noldus® Information Technology, Wageningen, Netherlands). Total experimental duration was 20 min with the first minute of data discarded to account for disturbance and acclimation to experimental setup.

2.4. Data analysis

All analyses were performed in R language (*R: The R Project for Stanley et al., 2007*) using the R packages dplyr (version 1.1.1) (Wickham et al., 2023), ggplot2 (version 3.4.2) (Wickham et al., 2021).

The concentration of fluoxetine and its principal metabolite, norfluoxetine, in our mesocosms were modeled using a combined approach encompassing both consecutive degradation modeling and mass balance principles (Petrucci, 2007). Both fluoxetine and norfluoxetine decay were modeled with first-order kinetics and iterations for each day and initial concentration (see Supplementary materials).

To allow for a graphical display of time-dependent treatment effects, we use Principal Response Curves (PRC) models, based on redundancy analysis (RDA), adjusted for the response over time as observed in a control (Van den Brink and Braak, 1999). The response of the control Limnotron was taken as a reference and its principal curve is set to zero. The water quality variables used in this multivariate analysis are: dissolved oxygen (DO), electrical conductivity (EC), pH, dissolved nutrients (NO_2^-, NO_3^-, NH_4^+ and PO_4^{3-}), particulate organic carbon (C) & particulate organic phosphorus (P), total chlorophyll-a concentration (total chl-a), and green photosynthetic yield (total yield). All variables were standardized using the scale function in R prior to PRC analysis.

The impact of fluoxetine and microplastics on phytoplankton biomass accumulation, zooplankton abundance and decomposition in the mesocosm experiment was visualized using the area under the curve (AUC) i.e. the accumulative chl-a concentration (μg/L over 103 days) for phytoplankton and zooplankton abundance of different treatments with spline interpolation method (*DescTools Package - RDocumentation*, 2023). LOESS smoothing with 95% confidence interval was applied to aid visualization of trends. Spearman's rank correlation a non-parametric analysis was used to test for the presence of non-monotonicity, calculating rho (ρ), a correlation coefficient, to measure the strength and direction of monotonic relationship between treatment and response (zooplankton abundance/phytoplankton biomass accumulation/decomposition rate). It ranges from −1 to 1, where −1 indicates a perfect negative monotonic relationship, +1, a perfect positive monotonic relationship, and 0, non-monotonic relationship. The significance of chronic effect of microplastics and fluoxetine on the aforementioned responses were further investigated using Generalized Additive Models (GAM). Our GAM model predicts a response variable based on two key predictors: a smoothed, non-linear function of fluoxetine concentration, and a linear term for microplastics. This dual approach allows the GAM model to effectively capture the nuanced interplay between these variables and their impact on the response variable. The GAM model is particularly effective in scenarios with complex, non-linear relationships and unknown patterns between variables, offering adaptable and accurate analysis (Hastie and Tibshirani, 1986).

Behavioral analysis profiles were set up and the distance moved (cm), velocity (cm/s), and duration of movement (s) of the individuals were analyzed in EthoVision® XT software automated tracking software and exported as an Excel spreadsheet into 30 s-time bins. Behavioral variables were analyzed using linear mixed-effects regression (lmer) models, using the package *lme4* (Bates et al., 2023), based on maximum likelihood, for each species separately. The factor "time bin" was used as a random factor to account for non-independence of time samples and to account for within trial time effects. Least squared means calculated using the 'lsmeans' function in R (Lenth, 2018) with Tukey's honest significant difference test was performed to determine differences between Limnotron treatments post-hoc.

3. Results

3.1. Water quality variables

We quantitatively modeled the actual exposure concentrations of fluoxetine and its metabolite norfluoxetine within the mesocosms (Fig. S1), because the environmental matrix and exposure concentrations often fell below the limit of quantification (<150–500 ng/L). We found a modeled decrease in fluoxetine levels, which plateaued to a relatively stable concentration after a 20-day period. Conversely, norfluoxetine, exhibited an exponential increase in concentration during the initial 20-day interval, reaching a peak concentration of 300 ng/L (i.e., for the highest initial fluoxetine initial concentration). Following this peak, nor-fluoxetine levels stabilized although maintaining higher concentrations than fluoxetine (see Fig. S1). Based on our model calculations, the effects driven by chemicals associated with microplastics per se (i.e., additives, plasticizers, monomers) are negligible (Supplementary materials, see: Calculations of worst-case styrene and PAH concentrations in the Limnotrons). Therefore, the effects presented in this study can be attributed to the particles themselves.

Immediately after the start of experiment (at T_1), there was low dissolved oxygen (1.5–2 mg/L) in treatments MP_FL3, MP_FL4, MP_FL5, MP_FL6 & MP_FL7 after which the dissolved oxygen in these treatments increased to a similar range (3–5 mg/L) as in MP_FL0, MP_FL1 & MP_FL2 (Figs. S2 and SI). Nitrate concentrations in all the treatments fluctuated throughout the experiment but all the treatments (including control) followed a similar trend until day 50, after which, NO_3^- among the Limnotron treatments diverged. Particulate organic carbon among all Limnotrons treatments (except for the initial decrease in MP_FL4) followed similar trends until day 75. Organic carbon concentration increased in Limnotrons with low fluoxetine + microplastics treatments i.e., MP_FL1 & MP_FL2 after day 80 relative to the control. Soon after the start of the experiment (between T_1 & T_6), a decrease in organic phosphorus (P) content in all the treatments (including control) was observed, after which P content in all the treatments increased. More specifically, after day 75, P content among different Limnotron treatments diverged with the highest P content in MP_FL7 and MP_FL4 Limnotron treatments. Total chlorophyll-a concentrations in the control were higher than the microplastics and microplastics + fluoxetine treatments (Fig. S2I).

The Principal Response Curve (PRC) analysis was used to show temporal trends in water quality variables compared to the control. During the 103-day exposure period (Fig. 2), all treatments exhibited varying positive and negative trends, with extremes becoming more pronounced as time progressed. The response variables dissolved oxygen (DO), pH, NO_2^-, and organic phosphorus (P) accounted for the largest contribution to the variation in the principal response variable. Photosynthetic yield, along with NO_3^- and NH_4^+, played a minor role in determining the overall response patterns. Additionally, DO, pH, and NO_2^- developed in opposite directions to photosynthetic yield, NO_3^-, and NH_4^+, indicating a negative correlation between these two groups of variables. The different nature of the relationships was apparent in the individual time series (Fig. S2) where up to week 6, the Limnotron treatments showed higher DO, pH, and NO_2^-, along with a lower organic P, NO_3^- and NH_4^+. Around week 7–8 (at 50 days), this pattern reversed, with increases in particulate organic phosphorus, photosynthetic yield, and dissolved nutrients (NO_3^-, NH_4^+), and decreases in DO and pH.

3.2. Phytoplankton biomass accumulation and zooplankton abundance

Phytoplankton biomass varied across treatments, with the control treatment having the highest chlorophyll-a concentration, followed by MP_FL2 and MP_FL3 treatments while MP_FL1, MP_FL4, and MP_FL5 showed the lowest concentrations (Fig. 3A). Initial dose-response relationship between treatments and phytoplankton biomass determined by Spearman's rank correlation ($\rho = -0.483$) showed a weak non-monotonic relationship, indicating that effects on phytoplankton differ along the exposure gradient. Further exploration using Generalized Additive Models (GAM) showed a strong significant effect of microplastics on phytoplankton (GAM, effect of microplastics, $p = 0.004$). Fluoxetine did not show an effect on phytoplankton biomass accumulation (GAM, effect of fluoxetine, $p = 0.76$). In addition, we observed that the interaction of MP and fluoxetine had significant effects on phytoplankton (GAM, MP × Fluoxetine, $p = 0.03$).

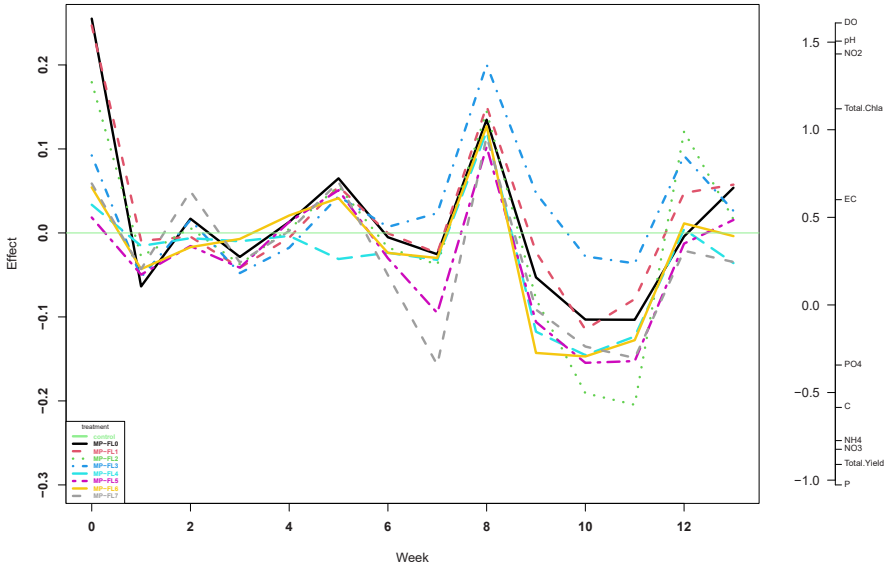

Fig. 2. Principal Response Curve (PRC) analysis shows the variation in water quality parameters over time of all treatments against control (corresponding to the y = 0 line) and determines the parameter that most contributes to variation between treatments (right -axis). Water quality parameters in PRC model: dissolved oxygen (DO), electrical conductivity (EC), pH, dissolved nutrients (NO_2^-, NO_3^-, NH_4^+ and PO_4^{3-}), Particulate organic carbon (C) & phosphorus (P), total chlorophyll-a concentration (total chl-a), and photosynthetic yield (total yield).

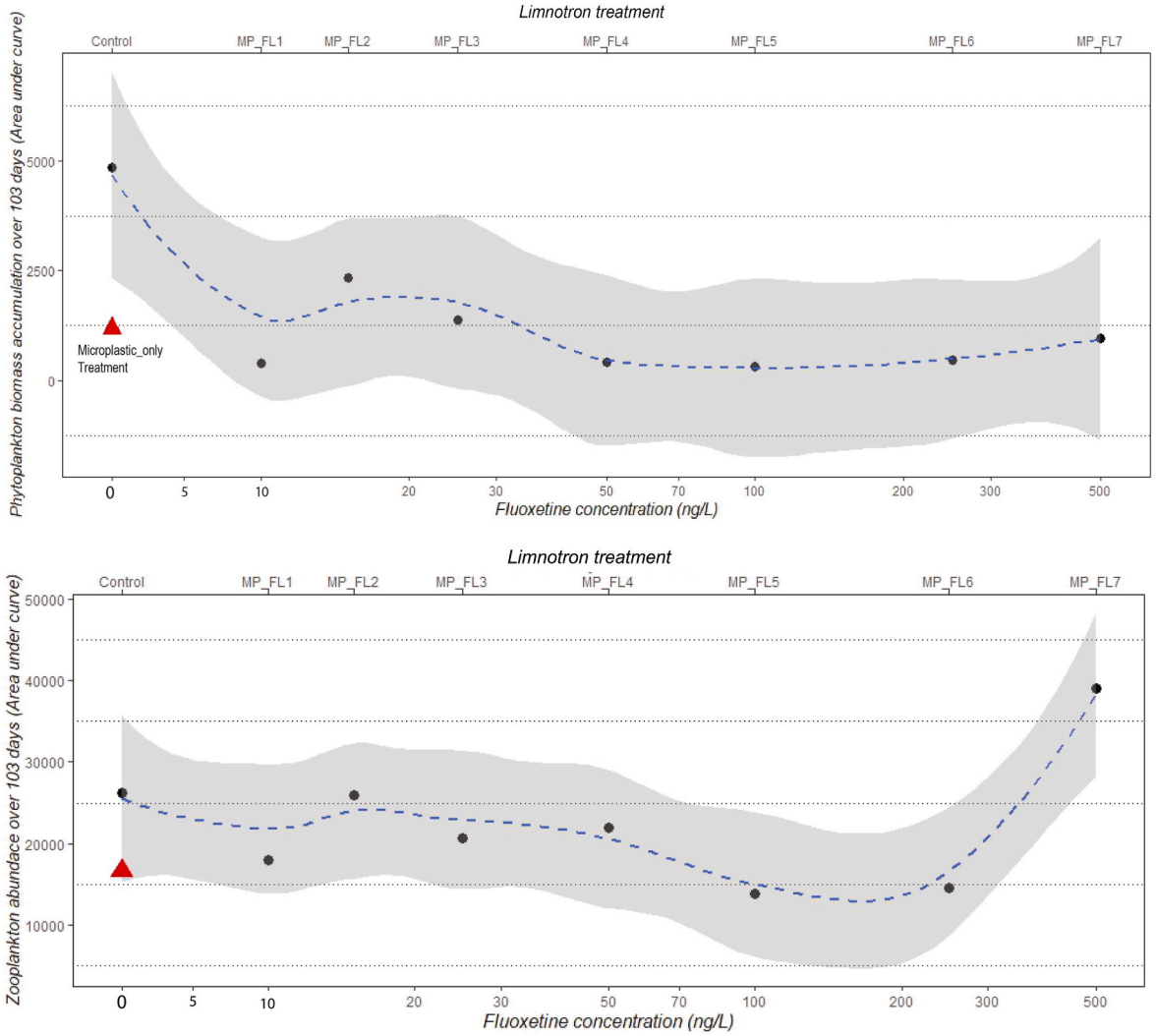

Fig. 3. Effect of fluoxetine in presence of microplastics on phytoplankton (Fig. 3A) and zooplankton (Fig. 3B) across fluoxetine (0–500 ng/L) and microplastics treatments indicated by the area under the curve (AUC) of phytoplankton biomass and the cumulative response of zooplankton abundance (individuals/L) for 103 days. Dashed lines depict trends in phytoplankton biomass accumulation and zooplankton abundance with gray shading denoting the 95% confidence interval. Red solid triangles represent microplastics-only treatments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

For the cumulative response of zooplankton abundance (Fig. 3B), we observed non-linear dose-response relationships characterized by both negative and positive effects across the gradient. This was reflected by a Spearman's rank correlation coefficient of $\rho = -0.1$, indicating a non-monotonic association between the dose and the observed response. Subsequent explorations employing GAM modeling revealed a moderate significant non-linear effect of fluoxetine on zooplankton abundance (GAM, effect of fluoxetine, $p = 0.02$). By contrast, the presence of microplastics did not exhibit an effect on zooplankton abundance (GAM, effect of microplastics, $p = 0.624$). In addition, a significant interaction between microplastics and fluoxetine was observed in affecting zooplankton populations (GAM, MP × Fluoxetine, $p = 0.038$).

Mesozooplankton and microzooplankton dynamics show all mesocosms started with similar zooplankton abundance (nearly zero individuals per liter). After two weeks of exposure the system started to be more dominated by microzooplankton. However, mesozooplankton commenced to thrive during the intermediate exposure time (from 21 to 80 days). Towards the end of the experiment both meso and microzooplankton followed similar patterns (Fig. S3). *Daphnia* sp. drove mesozooplankton trends with increased abundance across the experimental period (Fig. S4).

3.3. Decomposition

The decomposition rate (k_1) varied among the different locations within the mesocosms ranging between 0.0017 day^{-1} (MP_FL4, at the bottom) and 0.0117 day^{-1} (MP_FL1, at the middle of the water column (Fig. 4). Decomposition rates were highest at intermediate depths, followed by the bottom and top of the Limnotrons. The overall decomposition rate showed a non-linear response with a highest pick at MP_FL1 (0.0117 day^{-1}) and the lowest at MP_FL4 (0.0017 day^{-1}) which increased at treatments MP_FL6 (0.0089 day^{-1}) and MP_FL7 (0.0095 day^{-1}) (Fig. 4). The stabilization factor (S), a measure of the presence of recalcitrant organic carbon, in our experiment ranged between −0.109 (MP_FL6, bottom) and 0.185 (MP_FL2, middle). Similar to decomposition rate, stabilization factor was higher at the middle points compared to top and bottom compartments (Table S1).

Spearman's rank correlation coefficients (−0.35, −0.28, and −0.10) for the bottom, middle, and top compartments of Limnotrons, respectively, corroborate the existence of non-monotonic relationships between treatments and decomposition rates. This nonmonotonicity was further substantiated through GAM analysis, which revealed a moderate significant non-linear impact of fluoxetine on the decomposition rate,

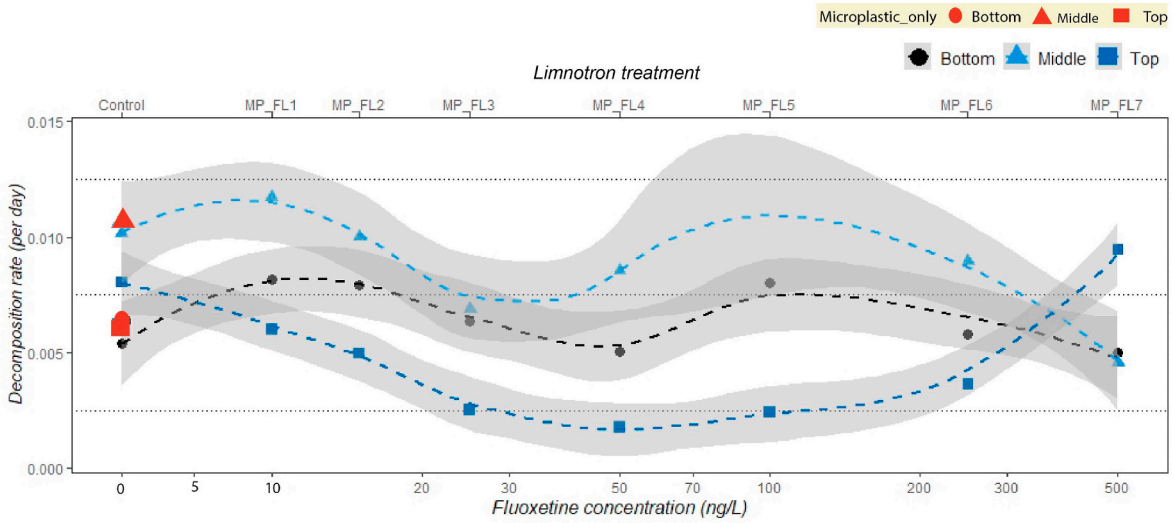

Fig. 4. Effect of fluoxetine in the presence of microplastics and microplastics only on decomposition rates over 90 days at different water depths (bottom, middle, top) and across a fluoxetine concentration gradient (0–500 ng/L). Solid symbols represent decomposition rate trends: circles (bottom, black), triangles (middle, green), and squares (top, blue). Dashed lines indicate trends in decomposition rate with microplastics + fluoxetine, with gray shading indicating the 95% confidence interval. Decomposition rate in Limnotron treatment with only microplastics is marked by red symbols (circle for bottom, triangle for middle, square for top). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

specifically near the water surface (GAM, fluoxetine effect, $p = 0.02$) and in the middle water column ($p = 0.048$). However, no significant effect of fluoxetine was observed near the sediment. In contrast, microplastics were found to have no significant impact on the decomposition rate in any of the studied water compartments (GAM, microplastics effect, $p > 0.05$). A significant interaction effect was detected between microplastics and fluoxetine on the decomposition rate near the water surface (GAM, MP × Fluoxetine, $p = 0.036$).

3.4. Behavioral assays

We performed behavioral assays on three species (*Chaoborus flavicans* larvae, *Daphnia magna*, and *Asellus aquaticus*) at days 5, 7, and 9 to assess their responses to a fluoxetine concentration gradient (0–500 ng/L) with microplastics (11.1 ng/L) and microplastics only (11.1 ng/L).

Limnotron treatments with fluoxetine + microplastics had a significant behavioral effect on *D. magna* (LMER: $p < 0.001$) which followed a non-monotonic response for all behavioral parameters. The lowest (MP-FL1) and the highest (MP-FL7) treatment significantly increased distance moved, mean velocity, and duration of movement ($p = 0.001$; Fig. 5A) whereas intermediate fluoxetine treatment (MP-FL2 to MP-FL6) significantly decreased the aforementioned parameters compared to the control ($p < 0.001$; Fig. 5A). By contrast, microplastics-only treatment (MP-FL0) did not show significant effect on D. magna behavior compared to the control (Fig. 5A).

Chaoborus flavicans showed significantly reduced movement in response to all treatments compared to the control ($p < 0.001$; Fig. 5B). The microplastics only treatment (MP_FL0) resulted in the least movement ($p < 0.001$), covering shorter distances and moving for less time (Fig. 5B). In contrast, *Asellus aquaticus* behavior was less affected by Limnotron treatments. Higher fluoxetine treatments in presence of microplastics (i.e., MP-FL5 and MP-FL7) had the most significant impact, reducing *A. aquaticus* distance moved, mean velocity and duration of movement ($p < 0.001$). Low dose (MP-FL1; $p = 0.0034$ and MP-FL2; $p = 0.046$) and intermediate fluoxetine concentrations + microplastics (i.e., MP-FL3; $p = 0.0011$ and MP-FL4; $p = 0.001$) reduced significantly *A. aquaticus* duration of movement. However, microplastics only (MP-FL0) treatment did not show behavioral effects on *A. aquaticus* compared to the control (Fig. 5C).

4. Discussion

Using long-term, near-natural, multi-trophic mesocosm systems, we discovered non-monotonic dose-response relationships in a broad range of biological parameters resulting from the exposure of fluoxetine in presence of polystyrene microplastics. These responses occurred over extended exposure periods (as evident from our mesocosm study), affecting zooplankton abundance, phytoplankton biomass, and microbial decomposition, as well as during acute exposures (as evident from our behavioral assays), influencing organismal behaviors. The observed non-monotonic dose responses were strongly influenced by exposure to fluoxetine for zooplankton abundance and decomposition, whereas phytoplankton standing crop seemed to be mostly governed by the presence of microplastics, with only a weak non-monotonic relationship due to the interaction between microplastics and fluoxetine exposure. Although the biological responses are interpreted in terms of exposure to the different fluoxetine concentrations, the observed results were likely from a combined effect of fluoxetine and nor-fluoxetine (the main degradation product). Nor-fluoxetine is an even more environmentally toxic compound due to its similar mode of action and longer half-life time (Yan et al., 2023). Our findings highlight the intricate interactions between fluoxetine, microplastics, and aquatic organisms, leading to non-monotonic dose response relationships where varying doses or exposure durations produce unexpected outcomes (Vandenberg et al., 2012).

4.1. Non-monotonic responses in the mesocosms

Phytoplankton biomass (standing crop) showed a weak non-monotonic response governed primarily by the presence of microplastics. Our results indicate that low concentrations of microplastics affect phytoplankton. This is in contrast with the broader scientific literature, which typically reports significant effects only at higher concentrations (Prata et al., 2018). Studies on the impact of microplastics and fluoxetine on phytoplankton reveal diverse responses depending on species and conditions. Polystyrene microplastics reduce population growth and chlorophyll concentrations in *Scenedesmus obliquus*, as well as in *Tetraselmis suecica* and *Amphora subtropica*, indicating a direct adverse impact on algae (Besseling et al., 2014; Raju et al., 2021). While microplastics might not induce toxicity at current

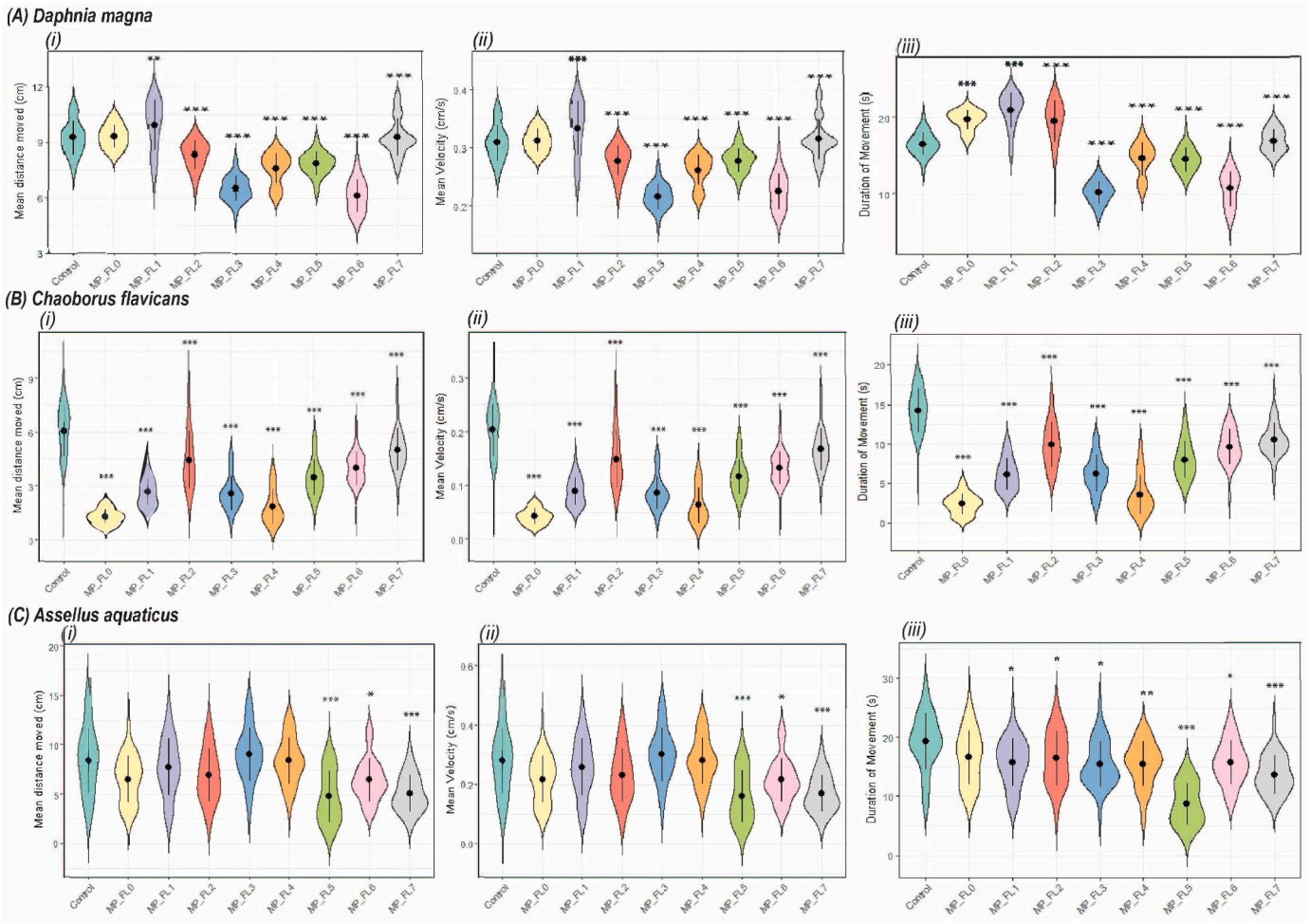

Fig. 5. Behavioral responses of different organisms (*Daphnia magna* in 5A, *Chaoborus flavicans* in 5B, and *Asellus aquaticus* in 5C) exposed to various Limnotron treatments (Control to MP_FL0-7) over 20 min. Each Figure assesses three parameters: (i) Distance moved (cm per interval), (ii) Mean velocity (cm/s), and (iii) Duration of movement (s per 30s interval). In violin plots, the black represents the median and the line length indicates the interquartile range. Statistical analysis used LMER with Tukey pairwise comparison between control and treatments. Asterisks indicate significance levels: *** for $p < 0.0001$, ** for $p < 0.001$, and * for $p < 0.05$.

environmental concentrations, they could disrupt phytoplankton populations by altering nutrient dynamics and predator-prey relationships (Prata et al., 2018; Hitchcock, 2022). In contrast, fluoxetine exposure (at 40–80 μg/L) reduced cell density and photosynthesis in phytoplankton (Feijão et al., 2020). However, phytoplankton have capacity to acclimatize over time, recovering photosynthetic efficiency and reducing oxidative stress even at high concentrations (500 and 1000 μg/L) (Xie et al., 2022). This adaptive response could explain that phytoplankton weak non-monotonic response may come from the dual effect of microplastics and initial shock of fluoxetine/nor-fluoxetine.

Contrary to the observed effects on phytoplankton, fluoxetine effects on zooplankton were evident at low concentrations (at ng/L range) in our mesocosm study. Zooplankton abundance over time showed a strong non-monotonic response to increasing doses of fluoxetine in the presence of microplastics. Fluoxetine exposure alters zooplankton behavior, reproduction, and growth leading to changes in zooplankton abundance (Fong & Ford, 2014). Our results align with previous studies that have demonstrated the potential of fluoxetine and other antidepressants to induce non-monotonic responses in several zooplankton species, even at low concentrations, in the ng/L range (Bossus et al., 2014; Fong & Ford, 2014; Rivetti et al., 2016; van der Most et al., 2023). However, our results bring unique insights to the field by examining the non-monotonic responses of zooplankton at the community level, particularly under environmental concentrations and over an extended exposure period of 103 days. This highlights that effects observed at individual species levels (also found in our behavioral assays) can propagate into population and community level responses.

Decomposition is a fundamental process which can affect ecosystem functioning through bottom-up processes. The microbial decomposition rate (k) and the stabilization factor (S) was higher in the middle of the water column indicating a microbial community that is capable of faster but less effective decomposition of plant material compared to the near surface water (top) and near sediment (bottom) compartments. Environmental factors (e.g. nutrients, temperature, oxygen concentration) and decomposer community composition influence decomposition (Ferreira et al., 2015; Gomes et al., 2018; Griffiths & Tiegs, 2016; Mori et al., 2023). Dissolved oxygen (DO; Fig. S2) in the middle of the water column was close to anoxic condition towards the end of the experiment, likely, DO levels in the bottom were even lower than in the middle, creating anaerobic conditions which are related to lower decomposition rates (Gomes et al., 2018). However, this does not explain the lower decomposition rate at the near water surface (top compartment). Several SSRIs including fluoxetine have been shown to induce antibacterial activity (bacteria and fungi) (Vasconcelos et al., 2022). This implies that the antimicrobial activity of fluoxetine was strongest to the microbial community inhabiting the near surface water compartment. Overall, the observed effects in our study on decomposition do not appear to be solely influenced by oxygen availability which is higher near the water

surface. A potential explanation could be that the decomposing microbial community, similar to the phytoplankton and zooplankton responses, follow a non-monotonic dose response. Consequently, this could hamper essential processes such as nutrient cycling and carbon sequestration, with knock-on effects on the functioning of aquatic ecosystems.

4.2. Non-monotonic responses in the bioassays

Incorporation of behavioral assays alongside a long-term mesocosm experiment allowed us to identify species-specific responses to fluoxetine and microplastics exposure. Responses towards fluoxetine are species specific due to deviating modes-of-action in invertebrates (Johnson & Sumpter, 2014) but also between vertebrates and invertebrates, due to differences in mode of action towards primary metabolites of fluoxetine (Stanley et al., 2007). *Daphnia magna* exhibited non-monotonic responses to fluoxetine (0–500 ng/L) in the presence of microplastics, with increased movement observed at lower (10–15 ng/L) and higher (500 ng/L) fluoxetine concentrations, similar to results in previous studies (Fong & Ford, 2014; Ford & Fong, 2016; Rivetti et al., 2016). However, our results differ from Nielsen & Roslev (2018), who observed varied swimming activity responses to fluoxetine concentrations. *Chaoborus flavicans* larvae also displayed increased movement (cm) and duration of movement (s) when exposed to microplastics and microplastics + fluoxetine (0–500 ng/L). With limited knowledge on the effect of anthropogenic contaminants on behavior of *C. flavicans*, it is challenging to compare our results to previous studies. However, exposure to a stressor (pCO2) increased movement (per min) and number of turns of *Chaoborus obscuripes* larvae (Kowalewska et al., 2020). Increased movement at high and low concentrations of fluoxetine may result in disruption of trophic interaction strength as a result of predator - prey mismatches in escape and pursuit velocities (South et al., 2019). Nonetheless, all reductions in movement and velocity compared to the control suggest a weakening of trophic interactions under any pollution scenario (South et al., 2019).

In contrast to *D. magna* and *C. flavicans*, *Asellus aquaticus* exhibited a limited response to the experimental treatments, with significant reductions in movement only observed when exposed to microplastics combined with higher concentrations of fluoxetine. *Asellus aquaticus* has been identified to have a variable response to numerous stressors, for example, *A. aquaticus* movement was decreased in presence of triazole fungicide tebuconazole and increased when exposed to the pyrethroid insecticide lambda-cyhalothrin (Bundschuh et al., 2012). The swimming speed of a functionally similar species, *Gammarus pulex*, increased with fluoxetine exposure (De Castro-Català et al., 2017), however, it reduced the swimming speed of fish species (Barry, 2013; Singer et al., 2016). Reduced activity *of A. aquaticus* at higher concentrations may also negatively impact decomposition rates in freshwater systems, albeit the general resilience of *A. aquaticus* to the concentrations tested indicates limited sub-lethal functional change.

5. Conclusions

Our study provides a long-term, mechanistic, understanding of multi-trophic biological responses across an environmentally realistic fluoxetine gradient in presence of polystyrene microplastics. Our approach also helps to understand the effects of nor-fluoxetine, which is more persistent in the aquatic environment. Chronic exposure resulted in significant non-monotonic dose effect responses in phytoplankton standing crop, zooplankton abundance, and the productivity of microbial communities involved in decomposition. Further investigation into potential mechanisms through behavioral experiments revealed similar non-monotonic responses in the zooplankton (*Daphnia magna*) and its predator species (*Chaoborus flavicans* larvae), while the benthic invertebrate (*Asellus aquaticus)* did not exhibit such responses. Our results thus demonstrate that short-term exposure effects may later develop into more ecosystem-level consequences as a result of chronic exposure.

CRediT authorship contribution statement

Nandini Vasantha Raman: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Berte M. Gebreyohanes Belay:** Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Investigation, Formal analysis, Data curation, Conceptualization. **Josie South:** Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Tarryn L. Botha:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Josephine Pegg:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Dumisani Khosa:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Lubabalo Mofu:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Gina Walsh:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Martine S. Jordaan:** Conceptualization, Investigation, Methodology, Writing – review & editing. **Albert A. Koelmans:** Writing – review & editing, Validation, Supervision, Formal analysis, Conceptualization. **Sven Teurlincx:** Writing – review & editing, Conceptualization. **Nico R. Helmsing:** Writing – review & editing, Methodology. **Nina de Jong:** Writing – review & editing, Investigation. **Ellen van Donk:** Writing – review & editing, Supervision. **Miquel Lürling:** Writing – review & editing, Supervision. **Victor Wepener:** Writing – review & editing, Conceptualization. **Tânia V. Fernandes:** Supervision, Writing – review & editing. **Lisette N. de Senerpont Domis:** Writing – review & editing, Validation, Supervision, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2024.124439.

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